Inhibine and Glucose Oxidase in Honey—A Review

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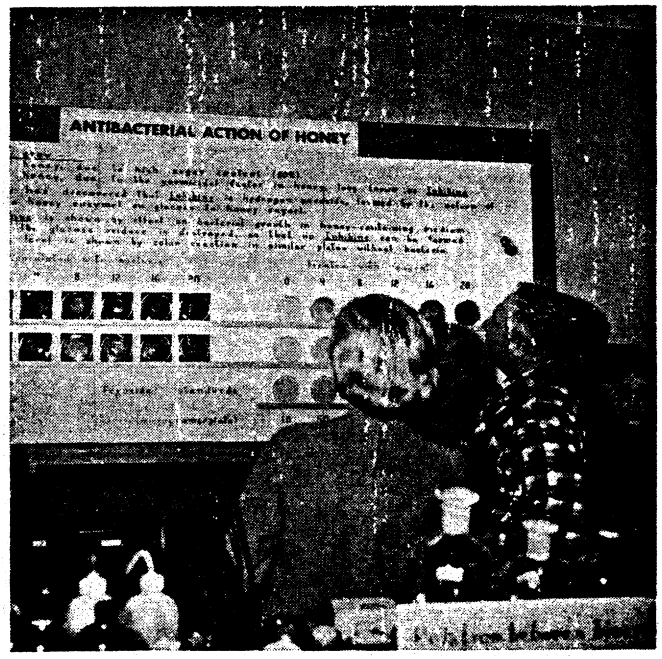

Figure 1. Visitors at laboratory open-house examine honey exhibit.

HONEY, AS THE FIRST sweetening agent known to man, has a long and interesting history. In addition to use for food, its origin, availability, and properties have been responsible for its wide variety of services to ancient and medieval man. Not the least of these was its use in medicine as a dressing for wounds and inflammations, both internal and external. For example, 30 percent of the 966 prescriptions in the ancient Egyptian Papyrus Ebers (ca. 1550 B.C.) used honey. Hindu, Greek and Roman medicine employed honey for a multitude of purposes. Such uses declined in the Middle Ages with the introduction of sugar and in this country today the medicinal use of honey is confined to "folk medicine." Modern reports may be found in the European literature however, attesting to the value of honey in treatment of wounds, burns, infections, urinary disorders and in cardiac therapy. Some of its effects may be ascribed to known components. One property, a definite antibacterial effect over and beyond the osmotic effect of the sugars, was reported by Dold, Du and Dziao (1937). Termed "inhibine," it was soon confirmed and is a recognized property of honey. The activity was reputed to be sensitive to heat and light, retained by bacterial filters and to pass through dialysis membrane. Activity was shown against many gram-positive and -negative bacteria. The standard assay (Dold and Witzenhausen 1955) involved the inoculation with *Staphylococcus aureus* of nutrient agar plates containing from 5 to 25 percent of honey, and after 24 hours incubation, observation of the concentration of honey not permitting growth.

In our work on the enzymes of honey, we demonstrated a glucose oxidase in honey (White, Subers and Schepartz 1962, 1963); Gauhe (1941) had shown that the pharyngeal glands of honey bees contained such an enzyme, but it had never been found in honey. As a typical glucose oxidase, the enzyme produces gluconolactone and hydrogen peroxide from glucose. In considering the implications of this, it was apparent that a possible explanation of the antibacterial activity of honey was available. It was then found that all the properties of inhibine may be explained by the production of hydrogen peroxide by the glucose oxidase of honey. If in the Dold-Witzenhausen assay procedure, either catalase or peroxidase plus an accepter is added, it completely destroys the antibacterial activity. If a honey is dialyzed to remove glucose, it shows no activity unless tested with glucose added. The rate of production of peroxide in the assay plates, measured photometrically, is closely related to the inhibine assay values of different honeys. The enzyme is active only in diluted honey. Thus, another ancient remedy, the use of honey as an antiseptic, is now seen to have a rational basis. At its original high density, bacteria are inhibited by honey's high sugar concentration; as hygroscopicity brings about dilution, the peroxide produced by the enzyme has a definite antibacterial effect. It is interesting that notatin or penicillin A which was encountered by the English during the wartime work on antibiotics, was found to be glucose oxidase.

Our experimental work on the subject is briefly described in an article in American Bee Journal (White, Subers and Schepartz 1962). Full technical details are included in another paper (White, Subers and Schepartz 1963).

For a recent laboratory open-house we prepared an exhibit (Fig. 1) showing actual bacteriological culture plates prepared with and without honey, and also similar plates containing a peroxide-measuring dye system. Portions of the exhibit are shown in Figures 2 and 3. In Figure 2 we see two series of six petri dishes, all inoculated with a culture of *S. aureus* and incubated for 24 hours. The plates were made with increasing amounts of honey in the agar medium, as shown. In the upper row, the honey was unheated and below, the plates contain corresponding amounts of a strongly heated honey. The bacterial growth, present on all of the lower series and the first two of the unheated plates, appears as an irregular white film. The clear appearance of the last

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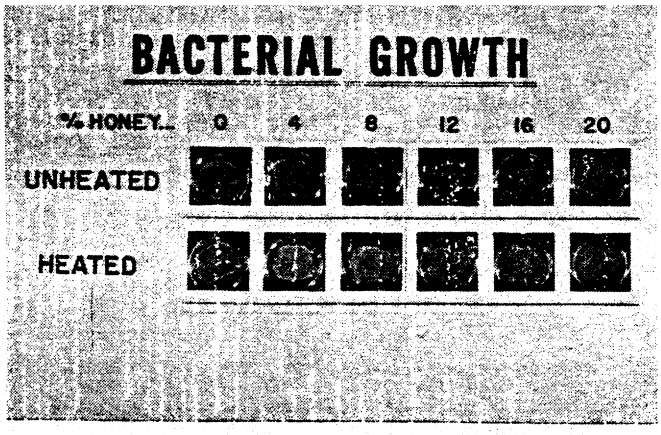

Figure 2. Culture plates containing indicated levels of unheated and heated honey, inoculated with S. AUREUS, and incubated 24 hours. Growth is seen as white film on plates.

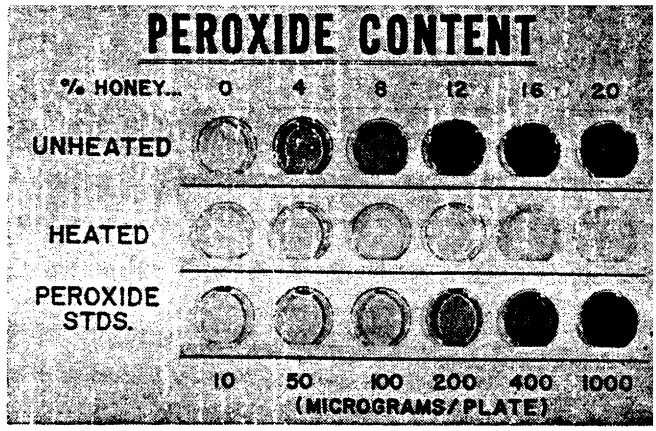

Figure 3. Plates corresponding to Fig. 2, not inoculated, but containing reagents to measure hydrogen peroxide content by color development. Bottom row of plates contains no honey: indicated amounts of hydrogen peroxide were added.

four plates in the upper row shows that the bacteria failed to grow. This assay shows that this honey has an inhibine number of 4, the number of clear plates in the series.

In Figure 3 the upper two rows of plates are similar to those in Figure 2, except that they were not inoculated with the bacteria. Instead, they contained a dye system (peroxidase-dianisidine) sensitive to hydrogen peroxide. Here the darker a plate appears the more peroxide it contains. In the lowest row, we have a set of six plates, with dye, to which measured amounts of peroxide were added, but no honey. By measuring the degree of darkening, compared with that from the known plates, the amount of peroxide in the honey plates was found. More than about 30 micrograms of peroxide per plate (about 0.0002%) prevented bacterial growth.

Subsequent research on inhibine and glucose oxidase in honey at this laboratory proceeded in two directions, study of the system in honey and fundamental research on the enzyme responsible.

The bacteriological assay previously described is too slow and cumbersome for routine assay of great numbers of honey samples, and also not very precise. A reproducible 1-hour chemical assay for peroxide accumulation in honey was developed (White and Subers 1963) and applied to 90 honey samples, of which 45 were also assayed by the old method. A study of the results showed that the inhibine number (0 to 5) was linearly related to the logarithm of the peroxide accumulation, as shown in Table 1.

TABLE 1

Relation Between Inhibine Number and Peroxide Accumulation[1]

Inhibine No.	Peroxide Accumulation[2]
0	less than 3.4
1	3.4 - 8.7
2	8.8 - 20.5
3	20.6 - 54.5
4	54.6 - 174
5	more than 174

[1]From White and Subers (1963).

[2]Micrograms hydrogen peroxide accumulated per gram honey in 1 hour under assay conditions.

Although diastase in honey originates from the bee, as does glucose oxidase, no correlation could be found between diastase number and peroxide accumulation in the 90 samples examined. Peroxide accumulation is not a measure of glucose oxidase alone, but rather of the difference between peroxide production by the enzyme and its destruction by various honey constituents, which can vary with floral source and other factors. One honey in particular—cotton honey—was found to be consistently very high in peroxide accumulation. The peroxide accumulation (inhibine) value of a honey is affected not only by glucose oxidase content (introduced by the bee) but by various minor peroxide-destroying components in the honey from sources such as nectar, pollen, yeasts, or by peroxide-destroying enzymes. It may be affected also by handling, storage and processing of the honey, and in some cases, by exposure to light. Some of these factors were studied further.

In the original work on inhibine it was reported to be heat sensitive. Subsequent workers generally confirmed this, though considerable variation among investigators was reported. We determined the effects of heat on peroxide accumulation (White and Subers 1964a). A wide variability appeared for 29 different honey samples, with a 70-fold difference in sensitivity being found in this limited survey. For six honeys, the relation between half-life of the peroxide accumulation system and temperature was determined and is seen in Figure 4. The previously known heat sensitivity of diastase and invertase is shown by the dashed lines. Only one of the six solid lines, which represent inhibine heat sensitivity, lies parallel with the diastase and invertase lines. This honey (HS 36) is a clover type. Though it responded to increasing heat similarly to the enzymes over the temperature range, it was much more sensitive to heat; in general, the peroxide accumulation system of honey is seen to be much more heat-sensitive than are the two enzymes named. It was concluded that the wide range of sensitivity of inhibine to heat found even in those few samples cast doubt on the utility of inhibine value as a means of detecting the previous exposure of honey to heat, as proposed by others.

Another property of inhibine that had been reported was light sensitivity. We also studied this (White and Subers

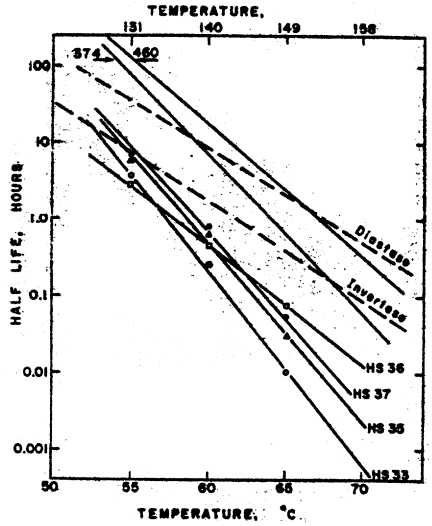

Figure 4. Effect of temperature on stability of honey peroxide accumulation system (inhibine).

1964b). The conflicting literature reports on this were explained when it was found that honeys varied tremendously in the sensitivity of their peroxide accumulation system to light. Some honeys may be exposed in thin layer to direct sunlight for a few minutes without effect, while working under ordinary laboratory lighting will be very destructive to inhibine in other honey types. Further examination of the destructive process showed that, contrary to destruction of simple enzymes by radiation, where ultraviolet light is responsible, visible light in the blue-green region (425-525mμ) is the most destructive of inhibine in honey. From a physical viewpoint, this means that a sensitizing material must be involved; indeed a heat- and light-stable, nonvolatile sensitizer was demonstrated to be present in honey most liable to light-destruction of their peroxide accumulation system. It was also noted that if the acidity of a honey is neutralized the honey is no longer light-sensitive.

Table 2 shows the effect on inhibine number of the light exposure of various samples of light-sensitive and of light-resistant honeys, and of mixtures of the two. It can easily be seen that the sensitizer from the sensitive honey causes its mixture with resistant honey to become sensitive. The effect is more easily seen when peroxide accumulation values are used rather than inhibine numbers.

In actual practice, we need not be overly concerned about the light-sensitivity of honey inhibine. In bulk, only a thin surface layer of a sensitive honey will suffer inhibine destruction, while within the bulk there would be insufficient light penetration for significant damage. In any case however, exposure of honey surfaces to direct sunlight for extended periods should be avoided if inhibine content is to be maintained.

Insufficient information is available on the photosensitivity of the inhibine of various kinds of honey, but it does appear that inhibine in fall-flower (aster-goldenrod) honey is very liable to light destruction.

Another direction of work has been a study of the enzyme glucose oxidase itself. Some of its properties are given in the first technical paper (White, Subers and Schepartz 1963); it was later partially purified and some general properties described (Schepartz and Subers 1964). It was found to differ from similar enzymes from other sources, in having a very high substrate concentration requirements. It is tempting to relate this high optimum substrate requirement to the normally high glucose concentration found in honey, but other factors appear to prevent any action of the enzyme in full-density honey. The enzyme is also quite specific for D-glucose. Only one other material tested (D-mannose) was oxidized, and that very slowly. In a subsequent paper (Schepartz 1965a) it was reported that the enzyme oxidized the β-form of D-glucose about six times faster than the closely related α-form. Additiontl kinetic work on the honey enzyme was reported in another paper (Schepartz 1965b).

It has been possible to report here only a portion of the experimental work carried out in our laboratory on honey inhibine and its relation to glucose oxidase and peroxide accumulation in diluted honey. Detailed information may be found in the cited articles.

Apart from peroxide, the principal product of the action in dilute honey of glucose oxidase is gluconolactone, or gluconic acid. All but two of 490 honey samples examined (White et al. 1962) contained lactone; gluconic acid is the principal acid of honey (Stinson et al. 1960). Since it is produced in ripened honey only at an extremely low rate, amounts such as found in honey must have been formed during ripening, while the nectar was being processed by the bees. The amounts of gluconic acid (gluconolactone) found in honey are consistent with the known rates of production in dilute honey and the amount of time normally available during ripening for its production. Perhaps the peroxide presumably present during ripening and the acidity produced at this time serve to preserve nectar until the sugar concentration becomes high enough to exert a preservative effect.

TABLE 2

Inhibine Assays of Irradiated Honeys[1]

Sensitive Honey	
Control unexposed	4
3 min. sun, original density	3
5 min. sun, original density	2
3 min. sun, and filter,[2] original density	4
1 hr. lab. lights, original density	2
10 min. Mercury Arc, original density	3
5 min. sun, pH 3.9, dilute	2
5 min. sun, pH 6.5 dilute	5
Resistant Honey	
Control, unexposed	5
5 min. sun, original density	5
5 min. sun, pH 6.5, diluted	5
Mixture	
Control, undiluted	4
5 min. sun, original density	1
5 min. sun, pH 6.5, diluted	5

[1]Data from White and Subers (1964b).

[2]Glass filter removed blue-green light.

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